METHOD FOR REPRODUCING IN VITRO THE RNA-DEPENDENT RNA
POLYMERASE AND TERMINAL NUCLEOTIDYL TRANSFERASE
ACTIVITIES ENCODED BY HEPATITIS C VIRUS (HCV)

DESCRIPTION

5   The present invention relates to the molecular
biology and virology of the hepatitis C virus (HCV).
More specifically, this invention has as its object the
RNA-dependent RNA polymerase (RdRp) and the nucleotidyl
terminal transferase (TNTase) activities produced by HCV,
10   methods of expression of the HCV RdRp and TNTase, methods
for assaying *in vitro* the RdRp and TNTase activities
encoded by HCV in order to identify, for therapeutic
purposes, compounds that inhibit these enzymatic
activities and therefore might interfere with the
15   replication of the HCV virus.

As is known, the hepatitis C virus (HCV) is the main
etiological agent of non-A, non-B hepatitis (NANB). It
is estimated that HCV causes at least 90% of post-
transfusional NANB viral hepatitis and 50% of sporadic
20   NANB hepatitis. Although great progress has been made in
the selection of blood donors and in the immunological
characterization of blood used for transfusions, there is
still a high number of HCV infections among those
receiving blood transfusions (one million or more
25   infections every year throughout the world).
Approximately 50% of HCV-infected individuals develop
cirrhosis of the liver within a period that can range
from 5 to 40 years. Furthermore, recent clinical studies
suggest that there is a correlation between chronic HCV
30   infection and the development of hepatocellular
carcinoma.

HCV is an enveloped virus containing an RNA positive
genome of approximately 9.4 kb. This virus is a member
of the Flaviviridae family, the other embers of which are
35   the flaviviruses and the pestiviruses. The RNA genome of
HCV has recently been mapped. Comparison of sequences
from the HCV genomes isolated in various parts of the

world has shown that these sequences can be extremely heterogeneous. The majority of the HCV genome is occupied by an open reading frame (ORF) that can vary between 9030 and 9099 nucleotides. This ORF codes for a single viral polyprotein, the length of which can vary from 3010 to 3033 amino acids. During the viral infection cycle, the polyprotein is proteolytically processed into the individual gene products necessary for replication of the virus. The genes coding for HCV structural proteins are located at the 5'-end of the ORF, whereas the region coding for the non-structural proteins occupies the rest of the ORF.

The structural proteins consist of C (core, 21 kDa), E1 (envelope, gp37) and E2 (NS1, gp61). C is a non-glycosylated protein of 21 kDa which probably forms the viral nucleocapsid. The protein E1 is a glycoprotein of approximately 37 kDa, which is believed to be a structural protein for the outer viral envelope. E2, another membrane glycoprotein of 61 kDa, is probably a second structural protein in the outer envelope of the virus.

The non-structural region starts with NS2 (p24), a hydrophobic protein of 24 kDa whose function is unknown. NS3, a protein of 68 kDa which follows NS2 in the polyprotein, is predicted to have two functional domains: a serine protease domain in the first 200 amino-terminal amino acids, and an RNA-dependent ATPase domain at the carboxy terminus. The gene region corresponding to NS4 codes for NS4A (p6) and NS4B (p26), two hydrophobic proteins of 6 and 26 kDa, respectively, whose functions have not yet been clarified. The gene corresponding to NS5 also codes for two proteins, NS5A (p56) and NS5B (p65), of 56 and 65 kDa, respectively.

Various molecular biological studies indicate that the signal peptidase, a protease associated with the endoplasmic reticulum of the host cell, is responsible for proteolytic processing in the non-structural region,

that is to say at sites C/E1, E1/E2 and E2/NS2. A virally-encoded protease activity of HCV appears to be responsible for the cleavage between NS2 and NS3. This protease activity is contained in a region comprising both part of NS2 and the part of NS3 containing the serine protease domain, but does not use the same catalytic mechanism. The serine protease contained in NS3 is responsible for cleavage at the junctions between S3 and NS4A, between NS4A and NS4B, between NS4B and NS5A and between NS5A and NS5B.

Similarly to other (+)-strand RNA viruses, the replication of HCV is thought to proceed *via* the initial synthesis of a complementary (-)-RNA strand, which serves, in turn, as template for the production of progeny (+)-strand RNA molecules. An RNA-dependent RNA polymerase (RdRp) has been postulated to be involved in both these steps. An amino acid sequence present in all the RNA-dependent RNA polymerases can be recognized within the NS5 region. This suggests that the NS5 region contains components of the viral replication machinery. Virally-encoded polymerases have traditionally been considered important targets for inhibition by antiviral compounds. In the specific case of HCV, the search for such substances has, however, been severely hindered by the lack of both a suitable model system of viral infection (e.g. infection of cells in culture or a facile animal model), and a functional RdRp enzymatic assay.

It has now been unexpectedly found that this important limitation can be overcome by adopting the method according to the present invention, which also gives additional advantages that will be evident from the following.

The present invention has as its object a method for reproducing in vitro the RNA-dependent RNA polymerase activity of HCV that makes use of sequences contained in the HCV NS5B protein. The terminal nucleotidyl transferase activity, a further property of the NS5B

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protein, can also be reproduced using this method. The
method takes advantage of the fact that the proteins
containing sequences of NS5B can be expressed in either
eukaryotic or prokaryotic heterologous systems: the
5   recombinant proteins containing sequences of NS5B, either
purified to apparent homogeneity or present in extracts
of overproducing organisms, can catalyse the addition of
ribonucleotides to the 3'-termini of exogenous RNA
molecules, either in a template-dependent (RdRp) or
10   template-independent (TNTase) fashion.

The invention also extends to a new composition of
matter, characterized in that it comprises proteins whose
sequences are described in SEQ ID NO: 1 or sequences
contained therein or derived therefrom. It is understood
15   that this sequence may vary in different HCV isolates, as
all the RNA viruses show a high degree of variability.
This new composition of matter has the RdRp activity
necessary to the HCV virus in order to replicate its
genome.

20   The present invention also has as its object the use
of this composition of matter in order to prepare an
enzymatic assay capable of identifying, for therapeutic
purposes, compounds that inhibit the enzymatic activities
associated with NS5B, including inhibitors of the RdRp
25   and that of the TNTase.

Up to this point a general description has been
given of the present invention. With the aid of the
following examples, a more detailed description of
specific embodiments thereof will now be given, in order
30   to give a clearer understanding of its objects,
characteristics, advantages and method of operation.

Figure 1 shows the plasmids constructs used for the
transfer of HCV cDNA into a baculovirus expression
vector.

35   Figure 2 shows the plasmids used for the in vitro
synthesis of the D-RNA substrate of the HCV RNA-dependent
RNA polymerase [pT7-7(DCoH)], and for the expression of

the HCV RNA-dependent RNA polymerase in E. coli cells [pT7-7(NS5B)], respectively.

Figure 3 shows a schematic drawing of (+) and (-) strands of D-RNA. The transcript contains the coding
5   region of the DCoH mRNA. The DNA-oligonucleotides a, b and c were designed to anneal with the newly-synthesized antisense RNA and the DNA/RNA hybrid was subjected to cleavage with RNase H. The lower part of the scheme depicts the expected RNA fragment sizes generated by
10   RNase digestion of the RNA (-) hybrid with oligonucleotides a, b and c, respectively.

DEPOSITS

E. Coli DH1 bacteria, transformed using the plasmids pBac 5B, pBac 25, pT7.7 DCoH and pT7.7NS5B - containing SEQ ID
15   NO:1; SEQ ID NO:2; the cDNA for transcription of SEQ ID NO:12; and SEQ ID NO:1, respectively, filed on May 9, 1995 with The National Collections of Industrial and Marine Bacteria Ltd. (NCIMB), Aberdeen, Scotland, UK. under access numbers NCIMB 40727, 40728, 40729 and 40730,
20   respectively.

EXAMPLE 1

Method of expression of HCV RdRp/TNTase in Spodoptera frugiperda clone 9 (Sf9) cultured cells.

Systems for expression of foreign genes in insect
25   cultured cells, such as Spodoptera frugiperda clone 9 (Sf9) cells infected with baculovirus vectors are known in the art (V. A. Luckow, Baculovirus systems for the expression of human gene products, (1993) Current Opinion in Biotechnology 4, pp. 564-572). Heterologous genes are
30   usually placed under the control of the strong polyhedrin promoter of the Autographa californica nuclear polyhedrosis virus of the Bombix mori nuclear polyhedrosis virus. Methods for the introduction of heterologous DNA in the desired site in the baculoviral
35   vectors by homologous recombination are also known in the art (D.R. O'Reilly, L. K. Miller, V.A. Luckow, (1992),

Baculovirus Expression Vectors-A   Laboratory Manual, W.
H. Freeman and Company, New York).

       Plasmid vectors pBac5B and pBac25 are derivatives of
a   derivative of pBlueBacIII (Invitrogen)   and   were
constructed for transfer of genes coding for NS4B and
other non-structural   HCV   proteins   in   baculovirus
expression vectors.   The plasmids are schematically
illustrated in figure 1 and their construction   is
described in detail in Example 8.   Selected fragments of
the cDNA corresponding to the genome of the HCV-BK
isolate (HCV-BK; Takamizawa, A., Mori, C., Fuke, I.,
Manabe, S., Murakami, S., Fujita, J., Onishi, E., Andoh,
T., Yoshida, I. and Okayama, H., (1991) Structure and
Organization of the Hepatitis C Virus Genome Isolated
from Human Carriers J. Virol., 65, 1105-1113) were cloned
under the strong polyhedrin promoter of the nuclear
polyhedrosis virus and flanked by sequences that allowed
homologous recombination in a baculovirus vector.

       In   order   to   construct   pBac5B,   a   PCR   product
containing the cDNA region encoding amino acids 2420 to
3010 of the HCV polyprotein and corresponding to the NS5B
protein (SEQ ID NO:1) was cloned between the BamHI and
HindIII sites of pBlue BacIII.   The PCR sense
oligonucleotide   contained   a   translation   initiation
signal, whereas the original HCV termination codon serves
for translation termination.

       pBac25 is a derivative of pBlueBacIII (Invitrogen)
where the cDNA region coding for amino acids 810 to 3010
of the HCV-BK polyprotein (SEQ ID NO:2) was cloned
between the NcoI and the HindIII restriction sites.

       Spodoptera frugiperda clone 9 (Sf9) cells and
baculovirus   recombination   kits   were   purchased   from
Invitrogen.   Cells were grown on dishes or in suspension
at 27°C in complete Grace's insect medium (Gibco)
containing   10%   foetal   bovine   serum   (Gibco).
Transfection, recombination, and selection of baculovirus
constructs   were   performed   as   recommended   by   the

manufacturer.   Two recombinant baculovirus clones, Bac25
and Bac5B, were isolated that contained the desired HCV
cDNA.

5
For protein expression, Sf9 cells were infected
either with the recombinant baculovirus Bac25 or Bac5B at
a density of 2×10^6 cells per ml in a ratio of about 5
virus particles per cell. 48-72 hours after infection,
the Sf9 cells were pelleted, washed once with phosphate
buffered saline (PBS) and carefully resuspended (7.5 x
10   10^7 cells per ml) in buffer A (10 mM Tris/Cl pH 8, 1.5 mM
$MgCl_2$, 10 mM NaCl) containing 1 mM dithiothreitol (DTT),
1 mM phenylmethylsulphonyl-fluoride (PMSF, Sigma) and 4
mg/ml leupeptin. All the following steps were performed
on ice: after swelling for 30 minutes, the cells were
15   disrupted by 20 strokes in a Dounce homogeniser using a
tight-fitting   pestle.   Glycerol,   as   well   as   the
detergents   Nonidet   P-40   (NP40)   and   3-[(3-
Cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate
(CHAPS), were added to final concentrations of 10% (v/v),
20   1% (v/v) and 0.5% /w/v), respectively, and the cellular
extract was incubated for a further hour on ice with
occasional agitation.   The nuclei were pelleted by
centrifugation for 10 minutes at 1000 x g, and the
supernatant was collected. The pellet was resuspended in
25   buffer A containing the above concentrations of glycerol
and detergents (0.5 ml per 7.5×10^7 nuclei) by 20
strokes in the Dounce homogeniser and then incubated for
one hour on ice.   After repelleting the nuclei, both
supernatants were combined, centrifuged for 10 minutes at
30   8000 x g and the pellet was discarded.   The resulting
crude cytoplasmic extract was used either directly to
determine the RdRp activity or further purified on a
sucrose gradient (see Example 5).

Infection of Sf9 cells with either the recombinant
35   baculovirus Bac25 or Bac5B leads to the expression of the
expected HCV proteins.   Indeed, following infection of
Sf9 cells with Bac25, correctly-processed HCV NS2 (24

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kDa), NS3 (68 kDa), NS4B (26 kDa), NS4A (6 kDa), NS5A (56
kDa) and NS5B (65 kDa) proteins can be detected in the
cell lysates by SDS-polyacrylamide gel electrophoresis
(SDS-PAGE) and immunostaining. Following infection of
5   Sf9 cells with Bac5B, only one HCV-encoded protein,
corresponding in size to authentic NS5B (65 kDa), is
detected by SDS-PAGE followed by immuno- or Coomassie
Blue staining.

EXAMPLE 2
10 Method of assay of recombinant HCV RdRp on a synthetic
RNA template/substrate.

The RdRp assay is based on the detection of labelled
nucleotides incorporated into novel RNA products. The *in
vitro* assay to determine RdRp activity was performed in a
15 total volume of 40 µl containing 1-5 µl of either Sf9
crude cytoplasmic extract or purified protein fraction.
Unfractionated or purified cytoplasmic extracts of Sf9
cells infected with Bac25 or Bac5B may be used as the
source of HCV RdRp. A Sf9 cell extract obtained from
20 cells infected with a recombinant baculovirus construct
expressing a protein that is not related to HCV may be
used as a negative control. The following supplements
are added to the reaction mixture (final concentrations):
20 mM Tris/Cl pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 25 mM KCl, 1
25 mM EDTA, 5-10 µCi [^{32}P] NTP of one species (unless
otherwise specified, GTP, 3000 Ci/mmol, Amersham, was
used), 0.5 mM each NTP (i.e. CTP, UTP, ATP unless
specified otherwise), 20 U RNasin (Promega), 0.5 µg RNA-
substrate (ca. 4 pmol; final concentration 100 nM), 2 µg
30 actinomycin D (Sigma). The reaction was incubated for
two hours at room temperature, stopped by the addition of
an equal volume of 2 x Proteinase K (PK, Boehringer
Mannheim) buffer (300 mM NaCl, 100 mM Tris/Cl pH 7.5, 1%
w/v SDS) and followed by half an hour of treatment with
35 50 µg of PK at 37°C. RNA products were PCA extracted,
precipitated with ethanol and analysed by electrophoresis
on 5% polyacrylamide gels containing 7M urea.

The RNA substrate we normally used for the assay (D-RNA) had the sequence reported in SEQ ID NO: 12, and was typically obtained by *in vitro* transcription of the linearized plasmid pT7-7(DCoH) with T7 polymerase, as described below.

Plasmid pT7-7(DCoH) (figure 2) was linearized with the unique *Bgl*II restriction site contained at the end of the DCoH coding sequence and transcribed *in vitro* with T7 polymerase (Stratagene) using the procedure described by the manufacturer.   Transcription was stopped by the addition of 5 U/10µl of DNaseI (Promega).   The mixture was incubated for a further 15 minutes and extracted with phenol/chloroform/ isoamylalcohol (PCA).   Unincorporated nucleotides were removed by gel-filtration through a 1-ml Sephadex G50 spun column.   After extraction with PCA and ethanol precipitation, the RNA was dried, redissolved in water and its concentration determined by optical density. at 260 nm.

As will be clear from the experiments described below, any other RNA molecule other than D-RNA, may be used for the RdRp assay of the invention.

The above described HCV RdRp assay gave rise to a characteristic pattern of radioactively-labelled reaction products: one labelled product, which comigrated with the substrate RNA was observed in all reactions, including the negative control.   This RNA species could also be visualised by silver staining and was thus thought to correspond to the input substrate RNA, labelled most likely by terminal nucleotidyl transferase activities present in cytoplasmic extracts of baculovirus-infected Sf9 cells.   In the reactions carried out with the cytoplasmic extracts of Sf9 cells infected with either Bac25 or Bac5B, but not of cells infected with a recombinant baculovirus construct expressing a protein that is not related to HCV, an additional band was observed, migrating faster than the substrate RNA.   This latter reaction product was found to be labelled to a

high specific activity, since it could be detected solely by autoradiography and not by silver staining. This novel product was found to be derived from the externally-added RNA template, as it was absent from

5   control reactions where no RNA was added. Interestingly, the formation of a labelled species migrating faster than the substrate RNA was consistently observed with a variety of template RNA molecules, whether containing the HCV 3'-untranslated region or not. The 399 nucleotide

10   mRNA of the liver-specific transcription cofactor DCoH (D-RNA) turned out to be an efficiently accepted substrate in our RdRp assay.

       In order to define the nature of the novel species generated in the reaction by the Bac25- or Bac5B-infected

15   cell extracts, we carried out the following series of experiments. (i) The product mixture was treated with RNAse A or Nuclease Pl. As this resulted in the complete disappearance of the radioactive bands, we concluded that both the labelled products were RNA molecules. (ii)

20   Omission from the reaction mixtures of any of the four nucleotide triphosphates resulted in labelling of only the input RNA, suggesting that the faster migrating species is a product of a polymerisation reaction. (iii) Omission of Mg^{2+} ions from the assay caused a complete

25   block of the reaction: neither synthesis of the novel RNA nor labelling of the input RNA were observed. (iv) When the assay was carried out with a radioactively labelled input RNA and unlabelled nucleotides, the labelled product was indistinguishable from that obtained under

30   the standard conditions. We concluded from this result that the novel RNA product is generated from the original input RNA molecule.

       Taken together, our data demonstrate that the extracts of Bac25- or Bac5B-infected Sf9 cells contain a

35   novel magnesium-dependent enzymatic activity that catalyses *de novo* RNA synthesis. This activity was shown to be dependent on the presence of added RNA, but

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independent of an added primer or of the origin of the
input RNA molecule. Moreover, as the products generated
by extracts of Sf9 cells infected with either Bac25 or
Bac5B appeared to be identical, the experiments just
5   described indicate that the observed RdRp activity is
encoded by the HCV NS5B protein.

EXAMPLE 3

Methods for the characterization of the HCV RdRp RNA
product

10   The following methods were employed in order to
elucidate the structural features of the newly-
synthesized RNA product. Under our standard
electrophoresis conditions (5% polyacrylamide, 7M urea),
the size of the novel RNA product appeared to be
15   approximately 200 nucleotides. This could be due to
either internal initiation of RNA transcription, or to
premature termination. These possibilities, however,
appeared to be very unlikely, since products derived from
RdRp assays using different RNA substrates were all found
20   to migrate significantly faster than their respective
templates. Increasing the temperature during
electrophoresis and the concentration of acrylamide in
the analytical gel lead to a significantly different
migration behaviour of the RdRp product. Thus, using for
25   instance a gel system containing 10% acrylamide, 7M urea,
where separation was carried out at higher temperature,
the RdRp product migrated slower than the input substrate
RNA, at a position corresponding to at least double the
length of the input RNA. A similar effect was observed
30   when RNA-denaturing agents such as methylhydroxy-mercury
(CH_3HgOH, 10 mM) were added to the RdRp products prior to
electrophoresis on a low-percentage/lower temperature
gel. These observations suggest that the RdRp product
possesses an extensive secondary structure.

35   We investigated the susceptibility of the product
molecule to a variety of ribonucleases of different
specificity. The product was completely degraded upon

treatment with RNase A. On the other hand, it was found
to be surprisingly resistant to single-strand specific
nuclease RNase T1. The input RNA was completely degraded
after 10 minutes incubation with 60 U RNase T1 at 22°C

5   and silver staining of the same gel confirmed that not
only the template, but also all other RNA usually
detectable in the cytoplasmic extracts of Sf9 cells was
completely hydrolysed during incubation with RNAse T1.
In contrast, the RdRp product remained unaltered and was

10   affected only following prolonged incubation with RNase
T1. Thus, after two hours of treatment with RNase T1,
the labelled product molecule could no longer be detected
at its original position in the gel. Instead, a new band
appeared that had an electrophoretic mobility similar to

15   the input template RNA. A similar effect was observed
when carrying out the RNAse T1 digestion for 1 hour, but
at different temperatures: at 22°C, the RdRp product
remained largely unaffected whereas at 37°C it was
converted to the new product that co-migrates with the

20   original substrate.

     The explanation for these observations is that the
input RNA serves as a template for the HCV RdRp, where
the 3'-OH is used to prime the synthesis of the
complementary strand by a turn-or "copy-back" mechanism

25   to give rise to a duplex RNA "hairpin" molecule,
consisting of the sense (template) strand to which an
antisense strand is covalently attached. Such a
structure would explain the unusual electrophoretic
mobility of the RdRp product on polyacrylamide gels as

30   well as its high resistance to single-strand specific
nucleases. The turn-around loop should not be base-
paired and therefore ought to be accessible to the
nucleases. Treatment with RNase T1 thus leads to the
hydrolysis of the covalent link between the sense and

35   antisense strands to yield a double-stranded RNA
molecule. During denaturing gel electrophoresis the two
strands become separated and only the newly-synthesized

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antisense strand, which should be similar in length to
the original RNA template, would remain detectable. This
mechanism would appear rather likely, especially in view
of the fact that this kind of product is generated by
5   several other RNA polymerases *in vitro*.

The following experiment was designed in order to
demonstrate that the RNA product labelled during the
polymerase reaction and apparently released by RNase T1
treatment exhibits antisense orientation with respect to
10   the input template. For this purpose, we synthesized
oligodeoxyribonucleotides corresponding to three separate
sequences of the input template RNA molecule (figure 2),
oligonucleotide a, corresponding to nucleotides 170-195
of D-RNA (SEQ ID NO: 3); oligonucleotide b, complementary
15   to nucleotides 286-309 (SEQ ID NO: 4); oligonucleotide c,
complementary to nucleotides 331-354 (SEQ ID NO: 5).
These were used to generate DNA/RNA hybrids with the
product of the polymerase reaction, such that they could
be subjected to RNase H digests. Initially, the complete
20   RdRp product was used in the hybridizations. However, as
this structure is too thermostable, no specific hybrids
were formed. The hairpin RNA was therefore pre-treated
with RNase T1, denatured by boiling for 5 minutes and
then allowed to cool down to room temperature in the
25   presence of the respective oligonucleotide. As expected,
exposure of the hybrids to RNase H yielded specific
cleavage products. Oligonucleotide a-directed cleavage
lead to products of about 170 and 220 nucleotides in
length, oligonucleotide b yielded products of about 290
30   and 110 nucleotides and oligonucleotide c gave rise to
fragments of about 330 and 65 nucleotides. As these
fragments have the expected sizes (see figure 3), the
results indicate that the HCV NS5B-mediated RNA synthesis
proceeds by a copy-back mechanism that generates a
35   hairpin-like RNA duplex.

EXAMPLE 4

Method of assay of recombinant HCV TNTase on a synthetic
RNA substrate

        The TNTase assay is based on the detection of
template-independent incorporation of labelled
5   nucleotides to the 3' hydroxyl group of RNA substrates.
The RNA substrate for the assay (D-RNA) was typically
obtained by *in vitro* transcription of the linearized
plasmid pT7-7DCOH with T7 polymerase as described in
Example 2. However, any other RNA molecule, other than
10   D-RNA, may be used for the TNTase assay of the invention.
        The in vitro assay to determine TNTase activity was
performed in a total volume of 40 µl containing 1-5 µl of
either Sf9 crude cytoplasmic extract or purified protein
fraction. Unfractionated or purified cytoplasmic
15   extracts of Sf9 cells infected with Bac25 or Bac5B may be
used as the source of HCV TNTase. An Sf9 cell extract
obtained from cells infected with a recombinant
baculovirus construct expressing a protein that is not
related to HCV may be used as a negative control. The
20   following supplements are added to the reaction mixture
(final concentrations): 20 mM Tris/Cl pH 7.5, 5 mM $MgCl_2$,
1 mM DTT, 25 mM KCl, 1 mM EDTA, 5-10 µCi [^{32}P] NTP of one
species (unless otherwise specified, UTP, 3000 Ci/mmol,
Amersham, was used), 20 U RNasin (Promega), 0.5 µg RNA-
25   substrate (ca. 4 pmol; final concentration 100 nM), 2 µg
actinomycin D (Sigma). The reaction was incubated for
two hours at room temperature, stopped by the addition of
an equal volume of 2 x Proteinase K (PK, Boehringer
Mannheim) buffer (300 mM NaCl, 100 mM Tris/Cl pH 7.5, 1%
30   w/v SDS) and followed by half an hour of treatment with
50 µg of PK at 37°C. RNA products were PCA extracted,
precipitated with ethanol and analysed by electrophoresis
on 5% polyacrylamide gels containing 7M urea.
        EXAMPLE 5
35 Method for the purification of the HCV RdRp/TNTase by
sucrose gradient sedimentation

A linear 0.3-1.5 M sucrose gradient was prepared in buffer A containing detergents (see Example 1). Up to 2 ml of extract of Sf9 cells infected with Bac5B or Bac25 (corresponding to about 8 x 10^7 cells) were loaded onto a

5   12 ml gradient. Centrifugation was carried out for 20 hours at 39000 x g using a Beckman SW40 rotor. 0.5 ml fractions were collected and assayed for activity. The NS5B protein, identified by western blotting, was found to migrate in the density gradients with an unexpectedly

10   high sedimentation coefficient. The viral protein and ribosomes were found to co-sediment in the same gradient fractions. This unique behaviour enabled us to separate the viral protein from the main bulk of cytoplasmic proteins, which remained on the top of the gradient. The

15   RdRp activity assay revealed that the RdRp activity co-sedimented with the NS5B protein. A terminal nucleotidyl transferase activity (TNTase) was also present in these fractions.

EXAMPLE 6

20   Method for the purification of the HCV TNTase/RdRp from Sf9 cells

Whole cell extracts are made from 1 g of Sf9 cells infected with Bac5B recombinant baculovirus. The frozen cells are thawed on ice in 10 ml of buffer containing 20

25   mM Tris/HCl pH 7.5, 1 mM EDTA, 10 mM DTT, 50% glycerol (N buffer) supplemented with 1 mM PMSF. Triton X-100 and NaCl are then added to a final concentration of 2% and 500 mM, respectively, in order to promote cell breakage. After the addition of $MgCl_2$ (10 mM) and DNase I (15

30   µg/ml), the mixture is stirred at room temperature for 30 minutes. The extract is then cleared by ultracentrifugation in a Beckman centrifuge, using a 90 Ti rotor at 40,000 rpm for 30 minutes at 4° C. The cleared extract is diluted with a buffer containing 20 mM

35   Tris/HCl pH 7.5, 1 mM EDTA, 10 mM DTT, 20% glycerol, 0.5% Triton X-100 (LG buffer) in order to adjust the NaCl concentration to 300 mM and incubated batchwise with 5 ml

of DEAE-Sepharose Fast Flow, equilibrated in LG buffer
containing 300 mM NaCl. The matrix is then poured into a
column and washed with two volumes of the same buffer.
The flow-through and the first wash of the DEAE-Sepharose

5   Fast Flow column is diluted 1:3 with LG buffer and
applied onto a Heparin-Sepharose CL6B column (10 ml)
equilibrated with LG buffer containing 100 mM NaCl. The
Heparin-Sepharose CL6B is washed thoroughly and the bound
proteins are eluted with a linear 100 ml gradient, from

10 100 mM to 1M NaCl in buffer LG. The fractions containing
NS5B, as judged by silver- and immuno-staining of SDS-
PAGE, are pooled and diluted with LG buffer in order to
adjust the NaCl concentration to 50 mM. The diluted
fractions are subsequently applied to a Mono Q-FPLC

15 column (1 ml) equilibrated with LG buffer containing 50
mM NaCl. Proteins are eluted with a linear gradient (20
ml) from 50 mM to 1M NaCl in LG buffer. The fractions
containing NS5B, as judged by silver- and immuno-staining
of SDS-PAGE, are pooled and dialysed against LG buffer

20 containing 100 mM NaCl. After extensive dialysis, the
pooled fractions were loaded onto a PoyU-Sepharose CL6B
(10 ml) equilibrated with LG buffer containing 100 mM
NaCl. The PoyU-Sepharose CL6B was washed thoroughly and
the bound proteins were eluted with a linear 100 ml

25 gradient, from 100 mM to 1M NaCl in buffer LG. The
fractions containing NS5B, as judged by silver- and
immuno-staining of SDS-PAGE, are pooled, dialysed against
LG buffer containing 100 mM NaCl and stored in liquid
nitrogen prior to activity assay.

30   Fractions containing the purified protein NS5B were
tested for the presence of both activities. The RdRp and
TNTase activities were found in the same fractions.
These results indicate that both activities, RNA-
dependent RNA polymerase and terminal ribonucleotide

35 transferase are the functions of the HCV NS5B protein.
    We tested the purified NS5B for terminal nucleotidyl
transferase activity with each of the four ribonucleotide

triphosphates at non-saturating substrate concentrations. The results clearly showed that UTP is the preferred TNTase substrate, followed by ATP, CTP and GTP irrespective of the origin of the input RNA.

5 EXAMPLE 7

Method of assay of recombinant HCV RdRp on a homopolymeric RNA template

Thus far we have described that HCV NS5B possesses an RNA-dependent RNA polymerase activity and that the

10 synthesis of complementary RNA strand is a template-primed reaction. Interestingly, using unfractionated cytoplasmic extracts of Bac5B or Bac25 infected Sf9 cells as a source of RdRp we were not able to observe complementary strand RNA synthesis that utilized an

15 exogenously added oligonucleotide as a primer. We reasoned that this could be due to the abundant ATP-dependent RNA-helicases that would certainly be present in our unfractionated extracts. We therefore wanted to address this question using the purified NS5B.

20 First of all, we wanted to establish whether the purified NS5B polymerase is capable of synthesizing RNA in a primer-dependent fashion on a homopolymeric RNA template: such a template should not be able to form intramolecular hairpins and therefore we expected that

25 complementary strand RNA synthesis be strictly primer-dependent. We thus measured UMP incorporation dependent on poly(A) template and evaluated both oligo(rU)$_{12}$ and oligo(dT)$_{12-18}$ as primers for the polymerase reaction. Incorporation of radioactive UMP was measured as follows.

30 The standard reaction (10 -100 µl) was carried out in a buffer containing 20 mM Tris/HCl pH 7.5, 5 mM MgCl$_2$, 1 mM DTT, 25 mM KCl, 1 mM EDTA, 20 U RNasin (Promega), 1 µCi [^{32}P] UTP (400 Ci/mmol, Amersham) or 1 µCi [^{3}H] UTP (55 Ci/mmol, Amersham), 10 µM UTP, and 10 µg/ml poly(A) or

35 poly(A)/oligo(dT)$_{12-18}$. Oligo(U)$_{12}$ (1µg/ml) was added a primer. Poly A and polyA/oligodT$_{12-18}$ were purchased from Pharmacia. Oligo(U)$_{12}$ was obtained from Genset. The final

NS5B enzyme concentration was 10-100 nM. Under these conditions the reaction procedeed linearly for up to 3 h hours. After 2 hours of incubation at 22_, the reaction was stopped by applying the samples to DE81 filters (Whatman), the filters washed thoroughly with 1M Na_2HPO_4/NaH_2PO_4, pH 7.0, rinsed with water, air dried and finally the filter-bound radioactivity was measured in a scintillation ß-counter. Alternatively, the in vitro-synthesized radioactive product was precipitated by 10% trichloroacetic acid with 100 µg of carrier tRNA in 0.2 M sodium pyrophosphate, collected on 0.45-µm Whatman GF/C filters, vacuum dried, and counted in scintilaltion fluid.

Although some $[^{32}P]UMP$ or $[^3H]UMP$ ncorporation was detectable even in the absence of a primer and is likely to be due to the terminal nucleotidyl transferase activity associated with our purified NS5B, up to 20% of product incorporation was observed only when oligo(rU)12 was included as primer in the reaction mixture. Unexpectedly, also oligo(dT)12-18 could function as a primer of poly(A)-dependent poly(U) synthesis, albeit with a lower efficiency.

Other template/primers suitable for measuring the RdRp activity of NS5B include poly(C)/oligo(G) or poly(C)/oligo(dG) in the presence of radioactive GTP, poly(G)/oligo(C) or poly(G)/oligo(dC) in the presence of radioactive CTP, poly(U)/oligo(A) or poly(U)/oligo(dA) in the presence of radioactive ATP, poly(I)/oligo(C) or poly(I)/oligo(dC) in the presence of radioactive CTP.

EXAMPLE 8

Method of Expression Of HCV RdRp/TNTase in E. Coli

The plasmid pT7-7(NS5B), described in Figure 2 and Example 8, was constructed in order to allow expression in E. coli of the HCV protein fragment having the sequence reported in SEQ ID NO 1. Such protein fragment contains the RdRp and the TNTase of NS5B, as discussed above. The fragment of HCV cDNA coding for the NS5B

protein was thus cloned downstream of the bacteriophage
T7 Ø10 promoter and in frame with the first ATG codon of
the phage T7 gene 10 protein, usig methods that are
known to the molecular biology practice and described in
5   detail in Example 8. The pT7-7(NS5B) plasmid also
contains the gene for the b-lactamase enzyme that can be
used as a marker of selection of E. coli cells
transformed with plasmid pT7-7(NS5B).

The plasmid pT7-7(NS5B) was then transformed in the
10   E. coli strain BL21(DE53), which is normally employed
for high-level expression of genes cloned into
expression vectors containing T7 promoter. In this
strain of E. coli, the T7 gene polymerase is carried on
the bacteriophage 1 DE53, which is integrated into the
15   chromosome of BL21 cells (Studier and Moffatt, Use of
bacteriophage T7 RNA polymerase to direct selective
high-level expression of cloned genes, (1986), J. Mol.
Biol. 189, p. 113-130). Expression from the gene of
interest is induced by addition of
20   isopropylthiogalactoside (IPTG) to the growth medium
according to a procedure that has been previously
described (Studier and Moffatt, 1986). The recombinant
NS5B protein fragment containing the RdRp is thus
produced in the inclusion bodies of the host cells.
25   Recombinant NS5B protein can be purified from the
particulate fraction of E. coli BL21(DE53) extracts and
refolded according to procedures that are known in the
art (D. R. Thatcher and A. Hichcok, Protein folding in
Biotechnology (1994) in "Mechanism of protein folding"
30   R. H. Pain EDITOR, IRL PRESS, p.229-255). Alternatively,
the recombinant NS5B protein could be produced as
soluble protein by lowering the temperature of the
bacterial growth media below 20_ C. The soluble protein
could thus be purified from lysates of E. coli
35   substantially as described in Example 5.
EXAMPLE 9
Detailed construction of the plasmids in figures

Selected fragments of the cDNA corresponding to the genome of the HCV-BK isolate (HCVBK) were cloned under the strong polyhedrin promoter of the nuclear polyhedrosis virus and flanked by sequences that allowed

5   homologous recombination in a baculovirus vector.

pBac5Bcontains the HCV-BK sequence comprised between nucleotide 7590 and 9366, and codes for the NS5B protein reported in SEQ ID NO: 1. In order to obtain this plasmid, a cDNA fragment was generated by PCR using

10   synthetic oligonucleotides having the sequences 5'-AAGGATCCATGTCAATGTCCTACACATGGAC-3' (SEQ ID NO: 6) and 5'-AATATTCGAATTCATCGGTTGGGGAGCAGGTAGATG-3' (SEQ ID NO: 7), respectively. The PCR product was then treated with the Klenow DNA polymerase, digested at the 5'-end with

15   BamHI, and subsequently cloned between the BamHI and SmaI sites of the Bluescript SK(+) vector. Subsequently, the cDNA fragment of interest was digested out with the restriction enzymes BamHI and HindIII and religated in the same sites of the pBlueBacIII vector (Invitrogen).

20   pBac25 is contains the HCV-BK cDNA region comprised between nucleotides 2759 and 9416 of and codes for amino acids 810 to 3010 of the HCV-BK polyprotein (SEQ ID NO: 2). This construct was obtained as follows. First, the 820bp cDNA fragment containing the HCV-BK sequence

25   comprised between nucleotides 2759 and 3578 was obtained from pCD(38-9.4) (Tomei L., Failla,C., Santolini, E., De Francesco, R. and La Monica, N. (1993) NS3 is a Serine Protease Required for Processing of Hepatitis C Virus PolyproteinJ. Virol., 67 , 4017-4026) by digestion with

30   NcoI and cloned in the NcoI site of the pBlueBacIII vector (Invitrogen) yielding a plasmid called pBacNCO.. The cDNA fragment containing the HCV-BK sequence comprised between nucleotides 1959 and 9416 was obtained from pCD(38-9.4) (Tomei et al., 1993) by digestion with

35   NotI and XbaI and cloned in the same sites of the Bluescript SK(+) vector yielding a plasmid called pBlsNX. The cDNA fragment containing the HCV-BK

sequence comprised between nucleotides 3304 and 9416 was obtained from pBlsNX by digestion with *SacII* and *HindIII* and cloned in the same sites of the pBlsNX plasmid, yielding the pBac25 plasmid.

5   pT7-7(DCoH) contains the entire coding region (316 nucleotides) of the rat dimerization cofactor of hepatocyte nuclear factor-1aå (DCoH; Mendel, D.B., Khavari, P.A., Conley, P.B., Graves, M.K., Hansen, L.P., Admon, A. and Crabtree, G.R. (1991) Characterization of

10   a Cofactor that Regulates Dimerization of a Mammalian Homeodomain Protein, *Science* 254, 1762-1767; GenBank accession number: M83740). The cDNA fragment corresponding to the coding sequence for rat DCoH was amplified by PCR using the synthetic oligonucleotide

15   Dpr1 and Dpr2 that have the sequence TGGCTGGCAAGGCACACAGGCT (SEQ ID NO: 8) and AGGCAGGGTAGATCTATGTC (SEQ ID NO: 9), respectively. The cDNA fragment thus obtained was cloned into the *SmaI* restriction site of the E. coli expression vector pT7-7.

20   The pT7-7 expression vector is ea derivative of pBR322 that contains, in addition to the ß-lactamase gene and the *Col* E1 orifgin of replication, the T7 polymerase promoter Ø10 and the translational start site for the T7 gene 10 protein (Tabor S. and Richerdson C. C. (1985) A

25   bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes, *Proc. Natl. Acad. Sci. USA* 82, 1074-1078).

        pT7-7(NS5B) contains the HCV sequence from nucleotide 7590 to nucleotide 9366, and codes for the NS5B protein

30   reported in SEQ ID NO: 1.

        In order to obtain this plasmid, a cDNA fragment was generated by PCR using synthetic oligonucleotides having the sequences 5'-TCAATGTCCTACACATGGAC-3' (SEQ ID NO: 10) and 5'-GATCTCTAGATCATCGGTTGGGGGAGGAGGTAGATGCC-3' (SEQ ID

35   NO: 11), respectively. The PCR product was then treated with the Klenow DNA polymerase, and subsequently ligated in the E. coli expression vector pT7-7 after linearizing

it with *EcoRI* and blunting its estremities with the
Klenow DNA polymerase. Alternatively, cDNA fragment was
generated by PCR using synthetic oligonucleotides having
the sequences 5'- TGTCAATGTCCTACACATGG-3' (SEQ ID NO:
5   13) and 5'-AATATTCGAATTCATCGGTTGGGGAGCAGGTAGATG-3' (SEQ
ID NO: 14), respectively. The PCR product was then
treated with the Klenow DNA polymerase, and subsequently
ligated in the E. coli expression vector pT7-7 after
linearizing it with *NdeI* and blunting its estremities
10   with the Klenow DNA polymerase.